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Metamorphosis in the Brachiopod *Terebratalia*: Evidence for a Role of Calcium Channel Function and the Dissociation of Shell Formation from Settlement

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Abstract. Larvae of *Terebratalia* will not undergo metamorphosis when maintained in a sterile environment unless they are 9–10 days old; under these conditions the frequency of normal metamorphosis is low. Four-day larvae are normally induced to metamorphose when they contact a suitable substrate. They will also undergo metamorphosis when they are treated with high K^+ seawater in the presence of Ca^{2+}. Additional experiments indicate that both substrate-induced and high K^+ seawater-induced metamorphosis may involve the function of voltage-dependent calcium channels.

Metamorphosis involves settlement of the larva followed by formation of the protegulum, the initial shell. In larvae that have been aged in a sterile environment and in larvae treated with high K^+ in seawater with low Ca^{2+}, partial metamorphosis takes place. Under these conditions the larva does not settle, however a protegulum forms. Substrate-induced metamorphosis does not occur in the absence of the distal end of the pedicle lobe of the larva which normally makes contact with the substrate, however, treatment with high K^+ seawater containing Ca^{2+} induces partial metamorphosis in these larvae. These experiments suggest that there are at least two centers in the larva that control metamorphosis.

Introduction

Adult articulate brachiopods are sessile organisms. The larvae of these animals do not feed; they disperse the species as a consequence of their behavior and their ability to settle and metamorphose at appropriate sites. The process of settlement and metamorphosis has been described for several species of articulate brachiopods (See Long and Stricker, 1991, for a review). These events are similar in all of the species examined (Fig. 1). The larva swims close to the substrate for a variable period of time. Settlement begins when the larva orients itself perpendicular to the substrate and becomes attached to it via a secretory product produced by the distal tip of its pedicle lobe. After attachment of the larva to the substrate, the mantle lobe flips so that instead of partially covering the pedicle lobe it now partially covers the apical lobe. In *Terebratalia transversa* the mantle lobe secretes a periostracum prior to flipping (Stricker and Reed, 1985a). Within one day after the mantle lobe moves to its new position, a protegulum (initial shell) containing calcium carbonate is deposited on the periostracum, which lies over the new outer surface of the mantle lobe and part of the pedicle lobe (Stricker and Reed, 1985b). By four days, the post settlement part of the pedicle lobe has secreted a cuticle (Stricker and Reed, 1985c). At an unknown time after settlement the endoderm makes contact with a region of the apical lobe ectoderm, and a mouth is formed giving the metamorphosed individual the capacity to feed.

Virtually nothing is known about the factors that are responsible for the induction of settlement and metamorphosis in brachiopods. In order to induce metamorphosis in *Terebratalia transversa*, investigators have introduced fragments of brachiopod shell, various pelecypod shells or *Sabellaria* tubes into dishes with the larvae. The larvae frequently settle and metamorphose on these substrates. Long and Stricker (1991) state that larvae of *Terebratalia transversa* will not settle on clean glass surfaces or on shell fragments from which diatoms and bacteria have been removed. Long (1964) is cited for this

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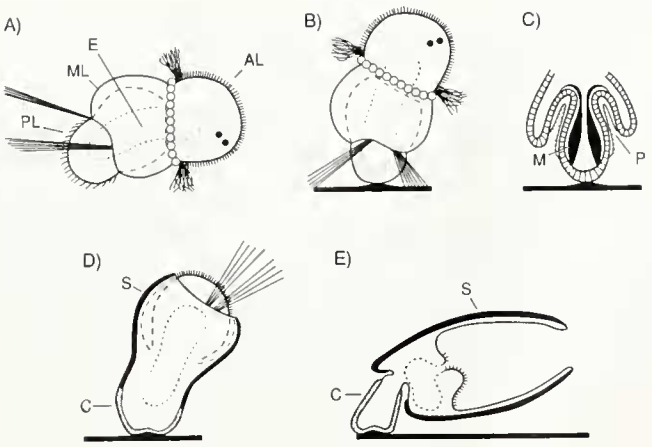

Figure 1. Diagrammatic view of swimming larva and larvae at various stages of substrate induced metamorphosis. (A) Side view of swimming larva. The larva is composed of three lobes. The apical lobe (AL) is at the anterior end of the larva. Behind the apical lobe there is a mantle lobe (ML) that partially covers the pedicle lobe (PL). The pedicle lobe comprises the posterior end of the larva. The apical lobe has a ciliary field that is responsible for larval locomotion. This lobe also has pigmented eye spots at its anterior end and a population of vesicular cells at its distal end bordering the cleft between the apical and mantle lobes. Setae extend from the distal end of the mantle lobe. An internal endodermal mass (E) is present. (B) Larva that has attached to the substrate by its pedicle lobe. (C) Longitudinal section showing the pedicle and part of the mantle lobe of a larva that has attached to a substrate. A periostracum (P) has formed externally between the upper part of the pedicle lobe and the inner surface of the mantle lobe. The inside of the larva has a pair of muscles (M) that insert in the mantle and pedicle lobes. The endodermal mass is not shown. (D) Side view of a larva where the mantle lobe has reversed and partially covers the apical lobe. Note the new position of the setae. A protegulum (S) has been laid down on the periostracum secreted by the mantle and pedicle lobes. The distal part of the pedicle lobe is forming a cuticle (C). (E) Side view of an individual that has completed metamorphosis. The endodermal region has formed a gut which is connected to the mantle cavity by a mouth. Shell has been added to the protegulum by the mantle.

information, however his dissertation does not make these statements.

In some animals the timing of metamorphosis appears to depend on internal signals that are only indirectly influenced by the external environment (*e.g.*, amphibia, White and Nicoll, 1981). In other organisms specific cues from the external environment play a necessary role in inducing metamorphosis (Burke, 1983a). Metamorphosis of larvae in several groups of marine invertebrate animals appears to fit this latter category. One way to distinguish between these two possibilities is to rear larvae in an environment that is devoid of the cues that are thought to induce metamorphosis and to see if metamorphosis will occur spontaneously. Larvae from several groups of animals that are competent to metamorphose will not undergo metamorphosis under these conditions (*e.g.*, Freeman, 1981, for hydrozoans). One aim of this study is to find out if this is the case for brachiopod larvae.

In those cases where specific chemical cues induce metamorphosis, the cues appear to activate receptor cells on the surface of the larva, and the receptor cells then activate a neuro-endocrine pathway that mediates metamorphosis (Morse, 1990). In some cases where metamorphosis is activated by an external chemical cue, this process can also be activated by depolarizing cells that are presumably part of the receptor or neuro-endocrine system that mediates metamorphosis by treating intact larvae with seawater containing excess K^+ (Yool *et al.*, 1986; Cameron *et al.*, 1989). Presumably the K^+ treatment activates membrane potential dependent ion channels such as Ca^{2+} or Na^+ channels. Another aim of this study is to provide evidence that settlement and metamorphosis in *Terebratalia transversa* occurs as a consequence of the opening of voltage-dependent calcium channels.

Metamorphosis involves a number of changes in the larva that occur at specific times after settlement. These changes appear to be coordinated. During the course of this work, larvae regularly have been observed that have not settled and have not reversed their mantle lobe but have secreted a protegulum. This observation indicates that different components of the metamorphic response can be dissociated from each other.

Materials and Methods

The biological material

Terebratalia transversa were dredged or collected by SCUBA at various subtidal localities near San Juan Island, Washington. The animals were maintained in aquaria with running seawater. Artificially inseminated cultures were prepared using the methods outlined in Strathman (1987). Because most *T. transversa* oocytes from a given female do not fertilize, cleavage stage embryos were picked out of the mass culture and washed in several changes of pasteurized seawater (PSW) with 100 units of Streptomycin per ml to dilute out the bacteria and inhibit their growth. Pasteurized seawater was prepared by filtering seawater through a 0.45 μM filter and heating it to 80–90°C. for 15 min followed by cooling and aeration. The embryos were reared in PSW with 100 units of streptomycin per ml in Falcon 1008 35 × 10 mm petri dishes until they had formed larvae. Streptomycin was always added to PSW immediately before use. The PSW with streptomycin and the petri dishes used were changed every other day. All experiments were carried out at 12–13°C.

For some experiments, four-day larvae were produced that had the distal tip of their pedicle removed. This operation was done by placing a larva in a Falcon plastic dish and using an electrolytically sharpened tungsten needle to cut through the pedicle lobe.

The induction of metamorphosis

Two methods were used for eliciting metamorphosis. Larvae were either exposed to natural substrates that induce metamorphosis or they were treated with seawater containing an excess of K^+ for a short time period to depolarize their cells. Natural substrate experiments were carried out in sterile flat bottom 10 × 15 mm Linbro sterile plastic dishes. One ml of cloth-filtered natural seawater was placed in the dishes and about 40 fragments of shell with external surface from a freshly smashed *T. transversa* or sand grains from the outside of a *Sabellaria* tube were added to the dish. About 20 four-day-old larvae were placed in the dish and the dish was incubated in the dark for 1 day. If 50% or more of the larvae had attached and reversed their mantle lobe, all of the animals were removed from the dish and the dish was set aside for subsequent experiments. Between 25 and 50% of the dishes were suitable for experimental purposes. When high K^+ seawater was used to induce metamorphosis, larvae were incubated in the high K^+ seawater for a defined period of time (30 min in most experiments), then washed in several changes of PSW to dilute out the K^+ and set aside in sterile Linbro dishes. They were assayed for metamorphosis at 24 h. In those experiments where larvae were treated with high K^+ in a modified seawater that lacked, or had a lower or a higher than normal amount of a given ion, the larvae were washed several times in the modified seawater before treatment with the high K^+ modified seawater. Table I gives the ionic composition of the different seawaters used.

Histological work

Larvae were fixed in 1% osmium in PSW in the cold for one hour, stored in 70% ethanol, dehydrated through an alcohol series, transferred to propylene oxide and embedded in an Epon equivalent. Sections were cut at 2 μM and stained with methylene blue.

Results

Can metamorphosis occur autonomously?

The first aim of this study was to find out if articulate brachiopod larvae would autonomously settle and undergo metamorphosis when reared in an environment with no external cues. Cohorts of 20 four-day-old larvae were set up in Linbro dishes in PSW with streptomycin. Each day of the experiment all of the larvae were examined for settlement and reversal of the mantle lobe and a cohort of larvae was examined with a compound microscope equipped with polarizing filters for shell mediated birefringence. Every other day, the larvae that were not used for birefringence studies were transferred to new sterile Linbro dishes with PSW containing streptomycin. The results of one of these experiments is presented in Table II.

Normal metamorphosis, settlement, reversal of the mantle lobe and formation of the protegulum was initiated only after larvae had been cultured in a sterile environment for at least 9 days; the percentage of larvae showing normal metamorphosis was low (29% at 10 days). Partial metamorphosis also took place in this experiment. Partial metamorphosis occurs when a larva does not settle and the mantle lobe is not reversed, but a birefringent mass forms in association with the mantle lobe (compare Fig. 2A and B). In a swimming larva, the eye spots in the apical lobe and the retractor muscles inside the pedicle lobe are birefringent. These can easily be distinguished from mantle lobe birefringence which is much stronger. The birefringent mass associated with the mantle lobe can be isolated by placing living larvae in 10% sodium hypochlorite in a 0.1 *M* phosphate buffer at pH 7 and

Table I

Ionic composition of artificial seawaters (mM)

Salt	Artificial SW	High K^+ SW	Na^+-free SW	High K^+ Na^+-free SW	Ca^{2+}-free SW	High K^+ Ca^{2+}-free SW	Mg^{2+}-free SW	High K^+ Mg^{2+}-free SW
NaCl	425.0	262.5	0.0	0.0	425.0	262.5	425.0	262.5
KCl	9.4	279.5	9.4	280.8	9.4	279.7	9.4	279.5
$CaCl_2 \cdot 2H_2O$	9.0	9.0	9.0	9.0	0.0	0.0	9.0	9.0
$MgCl_2 \cdot H_2O$	22.1	11.1	22.1	11.05	22.1	11.1	0.0	0.0
$MgSO_4$	25.6	12.8	25.6	12.8	25.6	12.8	0.0	0.0
$NaHCO_3$	2.1	1.1	0.0	0.0	2.1	1.1	2.1	1.1
TES buffer	10.0	5.0	10.0	5.0	10.0	5.0	10.0	5.0
Choline Cl			425.0	262.5				
$KHCO_3$			2.1	1.1				
Na_2SO_4							25.0	12.5

All seawaters had their pH adjusted to 7.9; 1*M* NaOH was used to adjust the pH except in Na^{2+}–free seawater where 1*M* KOH was used.

Table II

Settlement, normal metamorphosis, and partial metamorphosis in larvae reared under sterile conditions

Experiment number	Days in culture	Number settled	Mantle lobe reflected	Birefringent	Sample of swimming larvae	Percent birefringent
1	5	0/126			19	0
	6	0/102			18	0
	7	0/81			19	0
	8	0/59			17	0
	9	1/40	1	1	18	39
	10	6/21	6	3	15	87
2A	7	0/78			15	0
	8	0/61			14	0
	9	1/47	1	0	13	0
	10	0/31			15	40
	11	0/14			14	50
2B	7	0/76			14	0
	8	0/60			13	0
	9	0/44			13	0
	10	2/29	2	0	14	36
	11	1/14	1	1	13	54

changing the solution at frequent intervals until the organic material that makes up the larva is dissolved. One is left with a birefringent mass (Fig. 2C). When the birefringent mass was transferred to weak acid (0.1 *M* HCl), it effervesced as it dissolved indicating that it was composed of calcium carbonate. Sections through partially metamorphosed larvae showed that the calcium carbonate mass was located on the side of the mantle lobe that covered the pedicle lobe (Fig. 2D). This is the side of the mantle lobe on which the protegulum would have formed. The onset of partial metamorphosis was variable; In Experiment 1 in Table 2 it was observed as early as day nine. In a replica of this experiment with another batch of larvae (data not shown) partial metamorphosis was not observed until day 12 of culture. By the second day after the initial appearance of partial metamorphosis it was seen in 50–75% of the larvae.

A group of larvae may produce enough of a metabolite that induces metamorphosis during a two-day culture period to potentiate group metamorphosis. This possibility was examined in Experiment 2 (Table II). The onset of metamorphosis was compared for larvae from the same batch reared in groups of 16–20 in one ml dishes (Experiment 2A) or individually in one ml dishes (Experiment 2B). There was no difference in the onset of metamorphosis under these two culture conditions.

The ionic induction of metamorphosis: evidence for the involvement of voltage-dependent calcium channels

Four-day-old larvae were treated with high K^+ seawater (Table 1) for 30 min and set aside to see if they would metamorphose. The high K^+ seawater presumably depolarizes the cells of the larvae. In the high K^+ seawater the larvae stop swimming and show signs of sticking to the container. This treatment induces normal metamorphosis and partial metamorphosis in from 25–90% of the larvae, depending on the batch, when metamorphosis is assayed 24 h later (Fig. 3). Among the larvae that respond to high K^+ seawater, 40–90% undergo normal metamorphosis while the remainder undergo partial metamorphosis. Experiments where cohorts of larvae from the same batch were treated with high K^+ seawater for varying periods of time showed that a 5-min incubation in high K^+ seawater will not induce normal or partial metamorphosis while treatment with high K^+ seawater for 15, 30, or 45 min induces normal or partial metamorphosis with the same frequency. Treatment with high K^+ seawater for one or two hours reduces the percentage of larvae undergoing normal or partial metamorphosis (data not shown). While these experiments were done with a seawater with 280 m*M* K^+, artificial seawater with 114 m*M* K^+, 9 m*M* Ca^{2+} and slightly higher concentration of the other salts are equally effective (data not shown). These experiments suggest that the opening of voltage-dependent ion channels in appropriate cells may play a role in inducing metamorphosis.

The most common voltage-dependent ion channels are the calcium, sodium and potassium channels (Hille, 1984). The function of these different ion channels following membrane depolarization can be distinguished using the following criteria. (1) The movement of ions across cell membranes via these channels depends on their concentration in the external medium and the cytosol of

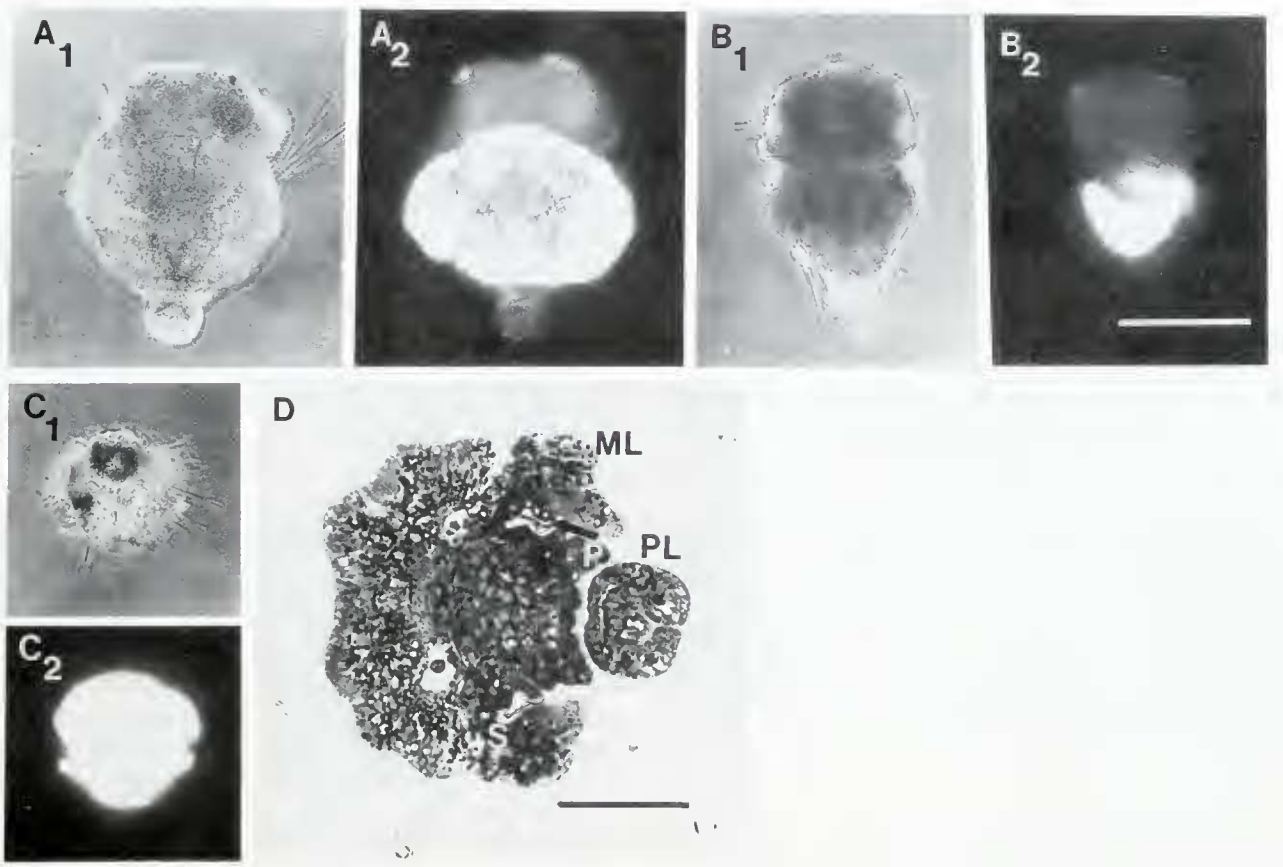

Figure 2. Photographs of larvae that have undergone normal or partial metamorphosis and the protegulum from a larva that has undergone partial metamorphosis. (A_1) Larva undergoing normal metamorphosis. The mantle lobe partly covers the apical lobe. Note the position of the setae and the eye spots of the apical lobe. (A_2) The same larva viewed with polarized light showing its birefringent protegulum and eye spots. (B_1) Partially metamorphosed larva. The mantle lobe partially covers the pedicle lobe and the setae have a position typical of a swimming larva. (B_2) Same larva viewed with polarized light showing the birefringent protegulum and eye spots. (C_1) Isolated protegulum from a partially metamorphosed larva. Note the setae embedded in the protegulum. (C_2) The same protegulum viewed with polarized light showing its birefringence. A–C are at the same magnification; the bar indicates 50 μM (D) Longitudinal section through a partially metamorphosed larva. Note the periostracum (P) between the mantle (ML) and the pedicle lobes (PL) and the space where the calcium carbonate (S) had been deposited. The bar indicates 100 μM

the cell. For example, in Ca^{2+}-free seawater the depolarization of cells should have no effect if internal Ca^{2+} levels must rise via calcium channels to initiate metamorphosis, (2) The function of specific ion channels is inhibited by channel blockers. For example, the ions Mg^{2+} and Co^{2+} and the drug nifedipine block calcium channel functions at concentrations that have no effect on sodium or potassium channel function.

Treatment of four-day-old larvae for 30 min with high K^+ in Na^+–free seawater induces normal and partial metamorphosis with the same frequency that normal and partial metamorphosis are induced in high K^+ seawater (Fig. 3A). Treatment of four-day-old larvae for 30 min with Na^+–free seawater, had no effect on metamorphosis (data not shown). Treatments of four-day-old larvae with high K^+ in Ca^{2+}–free seawater inhibited both normal and partial metamorphosis (Fig. 3B). These larvae were not damaged by the treatment in Ca^{2+}–free seawater because when some of these treated larvae were subsequently treated with high K^+ seawater, they were induced to undergo normal and partial metamorphosis.

The effects of the calcium channel blockers Co^{2+} and nifedipine on high K^+ seawater induced metamorphosis were tested (Fig. 3C, D). The Co^{2+} was prepared as a 360 mM $CoCl_2$ stock solution that was diluted appropriately with high K^+ seawater. The nifedipine was prepared as a 10 mM stock solution in ethanol and diluted appropriately in high K^+ seawater. When four-day-old larvae were inincubated for 30 min in high K^+ seawater with either 50 mM Co^{2+} or 0.2 mM nifedipine, normal and partial

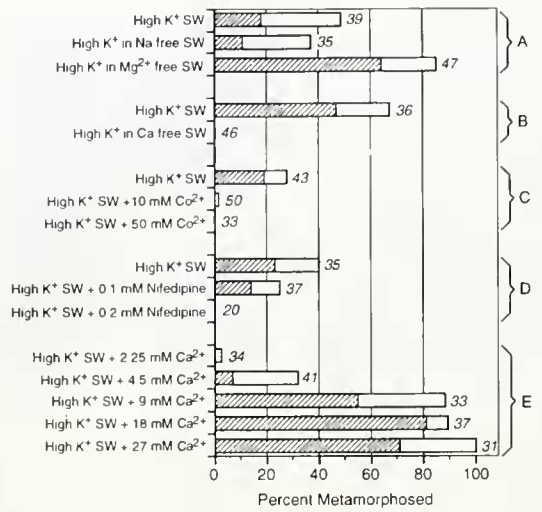

Figure 3. Histograms showing the effect of treatment with high K^+ in seawaters that lack Na^+, Ca^{2+}, or Mg^{2+}, in high K^+ seawater with different concentrations of Ca^{2+} and in high K^+ seawater with calcium channel blockers on metamorphosis. The hatched segment of each bar indicates the proportion of cases that underwent normal metamorphosis. The clear segment of the bar indicates the percentage of cases that underwent partial metamorphosis. The number of cases is indicated at the top of the bar. Experiments A–E were each done with a batch of larvae from a separate female.

metamorphosis was completely inhibited. When four-day-old larvae were incubated for 30 min in high K^+ seawater with the concentration of ethanol used to make the 0.2 m*M* nifedipine solution, metamorphosis was not inhibited (data not shown). The larvae that were treated with 50 m*M* Co^{2+} or 0.2 m*M* nifedipine were not damaged by these treatments because when some of these larvae were subsequently treated with high K^+ seawater in the absence of these calcium blockers, many of them were induced to undergo normal or partial metamorphosis. The calcium channel blocker Mg^{2+} is a normal constituent of seawater. Treatment of four-day-old larvae with high K^+ in Mg^{2+}–free saltwater induces both normal and partial metamorphosis in a higher percentage of cases than in high K^+ seawater (Fig. 3A). Treatment of four-day-old larvae for 30 min with Mg^{2+}–free seawater had no effect on metamorphosis (data not shown). When four-day-old larvae are treated with high K^+ seawater in the presence of the sodium channel blocker tetrodotoxin at a concentration of 20 n*M*, metamorphosis was not inhibited.

In an additional experiment, the effect of varying the external concentration of Ca^{2+} on high K^+ seawater mediated metamorphosis was tested by incubating four-day-old larvae for 30 min in an appropriate high K^+ seawater. The normal Ca^{2+} concentration in seawater is 9 m*M*. Concentrations of Ca^{2+} were used that were lower or higher than the normal concentration (Fig. 3E). At concentrations of 2.25 m*M* Ca^{2+} high K^+ seawater had almost no effect on metamorphosis. At concentrations between 4.5 and 9 m*M* Ca^{2+} the percentage of larvae undergoing metamorphosis increased; between 4.5 and 18 m*M* Ca^{2+} the proportion of larvae showing normal metamorphosis increased. Incubating four-day-old larvae for 30 min in artificial seawater with 18 or 27 m*M* Ca^{2+} had no effect on metamorphosis. The response to high K^+ seawater in the presence of elevated levels of Ca^{2+} was comparable to the responses to high K^+ seawater in the absence of the calcium channel blocker Mg^{2+}. In both cases there was a significant increase in the percentage of larvae that underwent normal as opposed to partial metamorphosis.

Does substrate induced metamorphosis involve putative voltage-dependent calcium channel function?

Substrate induced metamorphosis presumably involves a random walk on the part of the larva until it makes contact with a substrate bound inducer that elicits metamorphosis. These experiments were done to find out if inhibitors of calcium channel function, Co^{2+}, nifedipine, and Ca^{2+}-free seawater, also inhibit substrate induced metamorphosis and if Mg^{2+}-free seawater or high Ca^{2+} seawater, which facilitate calcium channel function, also facilitate substrate induced metamorphosis. Some of these experiments were not feasible. Incubation of larvae in Ca^{2+} or Mg^{2+}-free seawater for 24 h leads to a marked decrease in the adhesive bonds between cells, causing some cell dissociation. It was possible to do experiments in low Ca^{2+} seawater (2.25 m*M*). When larvae were incubated in seawater with 50 m*M* Co^{2+} or 0.2 m*M* nifedipine, both agents caused the larvae to stop swimming after a few hours rendering them unable to sample the substrate. After about 12 h the nifedipine began to come out of solution and form crystals at the air-water interface; as this happened, the larvae began to locomote again. It was possible to incubate larvae in seawater with 10 m*M* Co^{2+}; under these conditions larval locomotion slowed down but did not stop.

Four-day-old larvae were used for these experiments. Substrate induced metamorphosis differs from high K^+ induced metamorphosis in that the larvae either undergo natural metamorphosis, which was assayed 24 h after the experiment had been set up, or they retain their larval character. Very few cases of partial metamorphosis were observed. Over 100 larvae that were swimming after 24 h in the experiments without the channel blocker Co^{2+}, or low or high Ca^{2+} were examined; only three of these cases had a birefringent mantle lobe. Both 10 m*M* Co^{2+} and low Ca^{2+} seawater inhibited substrate induced metamorphosis (Fig. 4A, B). Treatment with Co^{2+} or low Ca^{2+} seawater for 24 h did not damage these larvae because when they were introduced into a dish with a substrate that would induce metamorphosis or treated with high

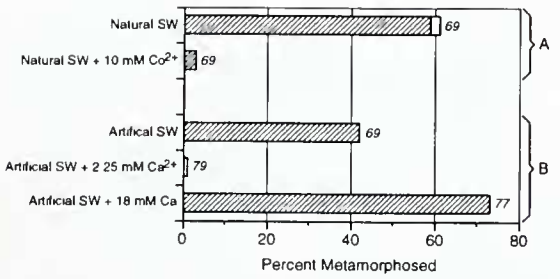

Figure 4. Histograms showing the effect of treatment with seawater with lower than normal or higher than normal concentrations of Ca^{2+} or the calcium channel blocker Co^{2+} on substrate induced metamorphosis. The hatched segment of each bar indicates the proportion of cases that underwent normal metamorphosis. The number of cases is indicated at the top of the bar. Experiments A and B were done with larvae from separate females.

K^+ seawater for 30 min, over half of these larvae underwent metamorphosis. The Co^{2+} and low Ca^{2+} seawater treatments did not alter the ability of the substrate in the Linbro dishes to induce metamorphosis. When the seawater with the Co^{2+} and the low Ca^{2+} seawater was removed from the wells and replaced by natural seawater and a batch of four-day larvae were added to the dish, metamorphosis was induced in more than 50% of the cases at 24 h. When larvae were placed in dishes with a substrate that would induce metamorphosis under conditions where the Ca^{2+} was higher than normal (18 m*M*), a higher proportion of larvae underwent metamorphosis than in dishes of seawater with the normal amount of Ca^{2+} (9 m*M*) (Fig. 4B). These experiments suggest that calcium channel function may also play a role in substrate induced metamorphosis.

The role of the pedicle lobe in metamorphosis

Prior to substrate induced metamorphosis the larva approaches the site where it will settle with its pedicle lobe, makes contact and adheres to the substrate with the distal end of its pedicle lobe. The possibility exists that there are substrate receptor cells in the pedicle that have to be activated to initiate metamorphosis. These may be the same cells that contain putative voltage-dependent calcium channels whose activation is necessary for metamorphosis.

In order to test this hypothesis four-day-old larvae were operated on to remove the distal portion of their pedicle lobe (Fig. 5), and two hours after the operations were completed they were tested to see whether or not they could undergo substrate induced or high K^+ seawater induced metamorphosis (Fig. 6). None of these larvae settled or reversed their mantle lobe. Settling should not be expected because the distal part of the pedicle lobe is missing, and reversal of the mantle lobe is also not expected because

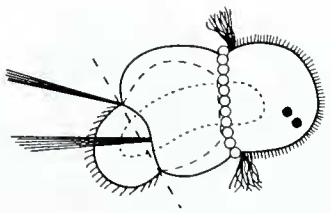

Figure 5. Diagram of a swimming larva showing the operation to remove the distal part of the pedicle lobe.

the retractor muscles that presumably play a role in moving the mantle lobe have been cut (see Discussion). All of the larvae with the distal end of the pedicle lobe missing swam around the Linbro dish among the substrates in a normal manner; at 24 h none of these larvae exhibited mantle birefringence indicating that substrate induced metamorphosis had not occurred. Many of the larvae with the distal end of the pedicle lobe missing that were treated with high K^+ seawater underwent partial metamorphosis when assayed at 24 h; however, the percentage of cases undergoing partial metamorphosis was not as high as it was for intact four-day-old larvae from the same batch. These results suggest that substrate receptors needed for metamorphosis are located in the pedicle lobe and that the removal of the distal region of the pedicle makes the larva unresponsive to substrate mediated cues, however, other cells with putative voltage-dependent calcium channels whose activation is necessary for metamorphosis are located in another region of the larva.

Can larvae that have undergone partial metamorphosis subsequently undergo normal metamorphosis?

It is not clear whether partial metamorphosis is the result of the activation of a metamorphic pathway or an epiphenomenon that is not related to metamorphosis. The strongest evidence that partial metamorphosis is related to normal metamorphosis is that both can be induced with high K^+ seawater and that both can occur spontaneously at the same time in aging larvae reared under

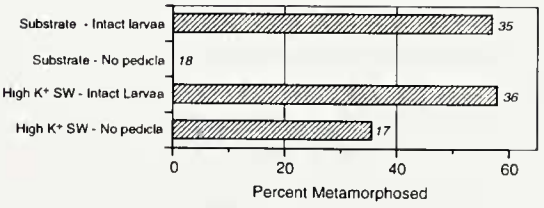

Figure 6. Histograms showing the effect of pedicle removal on substrate or high K^+ seawater induced metamorphosis. Metamorphosis was measured as larvae with birefringent mantle lobes. The number of cases is indicated at the top of the bar. The histograms combine data from two experiments on larvae from separate females.

sterile conditions. To better understand partial metamorphosis, experiments were done to find out if larvae that had undergone partial metamorphosis could respond to substrates that induce metamorphosis or high K^+ seawater by undergoing normal metamorphosis.

A large batch of four-day-old larvae were induced to undergo metamorphosis using high K^+ seawater. Under these conditions some of the larvae underwent normal metamorphosis, some underwent partial metamorphosis and some larvae had not metamorphosed when they were assayed one day later. The five-day-old larvae that had undergone partial metamorphosis and those that had not metamorphosed as a consequence of the high K^+ seawater treatment and five-day-old larvae from the same batch that had not previously been treated with high K^+ seawater were exposed to a substrate that would induce metamorphosis or high K^+ seawater with 18 m*M* Ca^{2+} which induces normal metamorphosis in a high percentage of cases. The results of these experiments (Fig. 7) show that larvae which have already undergone partial metamorphosis will not respond to a natural substrate that induces metamorphosis or to high K^+ seawater with 18 m*M* Ca^{2+} by settling or reversing their mantle. Reversal of the mantle may not be possible for these larvae because of the calcification of their mantle lobe; some movement of the lobe in these partially metamorphosed larvae is possible because when they are stimulated by prodding with a tungsten needle, the setae of the larvae take on a transient position perpendicular to the body. This same kind of movement takes place in normal larvae. There is no obvious reason why partially metamorphosed larvae should not be able to attach to the substrate. When larvae that had been treated with high K^+ seawater that did not undergo normal or partial metamorphosis were treated with a natural substrate that induces metamorphosis or high K^+ seawater with 18 m*M* Ca^{2+}, many of these larvae underwent natural or partial metamorphosis. The percentage of pretreated larvae that underwent metamorphosis was lower than the percentage of larvae metamorphosing that had not been pretreated with high K^+ seawater. This suggests that larvae which have been exposed to a metamorphic stimulus and do not respond, may either be less competent to respond to a metamorphic inducer and have been selected for via the pretreatment, or prior exposure to a metamorphic inducer may render these larvae less competent to respond to a subsequent metamorphic cue even though they have not undergone metamorphosis.

As part of this experiment some larvae that settled, but had not yet undergone mantle reversal, were gently removed from their substrate with a tungsten needle so that their pedicle lobe was not damaged and transferred to PSW in a sterile Linbro dish, while other larvae that had not undergone mantle reversal were left in place. All of the larvae that had not yet undergone mantle reversal and were not disturbed underwent normal metamorphosis by the next day (six cases). The larvae that were removed from their settlement site did not resettle but underwent partial metamorphosis (four out of six cases). This experiment suggests that settlement is necessary for normal metamorphosis.

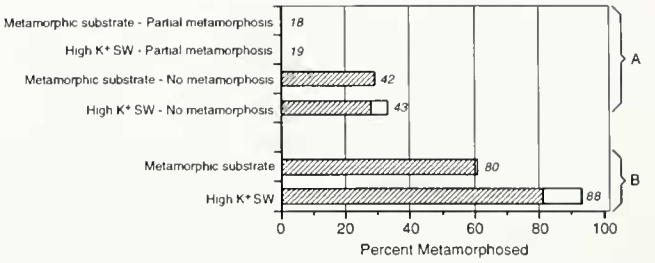

Figure 7. (A) Histograms showing the effect of pretreatment at four days of larvae with high K^+ seawater that either induced partial metamorphosis or no metamorphosis on the ability of these two categories of larvae to undergo metamorphosis after treatment with a metamorphosis substrate or high K^+ seawater at five days. (B) Histograms showing effect of treatment with a metamorphosis substrate or high K^+ seawater at five days on metamorphosis of larvae that had not been pretreated with high K^+ seawater at four days. The hatched segment of each bar indicates the proportion of cases that underwent normal metamorphosis. The clear segment of the bar indicates the percentage of cases that underwent partial metamorphosis. The number of cases is at the top of the bar.

Discussion

The role of voltage-dependent Ca^{2+} channels in metamorphosis

The following lines of evidence indicate that voltage-dependent calcium channels may play a role in metamorphosis: (1) Treatment of larvae with high K^+ seawater which presumably depolarizes the cells of the larva induces metamorphosis and treatment of larvae with high K^+ in Na^+-free seawater is just as effective in inducing metamorphosis, (2) Treatment of larvae with high K^+ in Ca^{2+}-free seawater inhibits metamorphosis, (3) Treatment of larvae with high K^+ in seawater with elevated Ca^{2+} levels or Mg^{2+}-free seawater increases the percentage of cases metamorphosing, (4) Treatment of larvae with high K^+ seawater in the presence of the calcium channel blockers Co^{2+} and Nifedipine inhibits metamorphosis. In order to make this work more convincing one would have to demonstrate electrophysiologically that target cells are not only depolarized but give an action potential which is typical of voltage-dependent Ca^{2+} channels and that Ca^{2+} moves into the target cells from the external environment during depolarization.

The identities of the target cells where voltage-dependent calcium channels function to mediate the metamorphic stimulus is not known. One possible target cell

candidate is a subset of cells in the larval nervous system. There is evidence that the nervous system receives and mediates the metamorphic stimulus in echinoid larvae (Burke, 1983b). Unfortunately, virtually nothing is known about the organization of the nervous system in articulate brachiopod larvae; however, nerve cell processes have been noted in ultrastructural studies done on these larvae for other purposes (Stricker and Reed, 1985a). Another possible set of target cells could be some of the cells that make up the surface epithelium of the larva (*e.g.*, the cells of the distal part of the pedicle lobe). After these cells receive a metamorphic stimulus, it could be transferred to other epithelial cells of the larva by epithelial conduction. There is evidence that epithelial conduction mediates the metamorphic stimulus in hydrozoans (Freeman and Ridgway, 1990).

Both substrate and high K^+ seawater induced metamorphosis appear to depend on calcium channel function. Substrate induced metamorphosis also depends on the pedicle lobe while high K^+ seawater induced metamorphosis does not. The simplest model that accounts for these results is that there is a substrate-induced metamorphosis receptor at the distal end of the pedicle lobe. When this is activated a metamorphic signal is sent from this site to cells outside of the distal region of the pedicle lobe that must have their putative voltage-dependent calcium channels activated in order to spread the metamorphic stimulus (Fig. 8). When the cells outside the distal region of the pedicle lobe are activated, they also send an inhibitory signal to the cells in the distal region of the pedicle lobe preventing them from responding to substrate mediated metamorphic cues (Fig. 7).

The significance of "partially metamorphosed" larvae

The partially metamorphosed larva is most probably the result of an abnormal metamorphic response. This larva is characterized as a larva that forms a protegulum in the absence of mantle reversal and settlement. Because the formation of a protegulum under these conditions probably renders the mantle lobe incapable of reversal and because the mantle lobe does not spread out to occupy a larger area as it does after reversal, this metamorphic response is probably maladaptive. I have made only limited attempts to look for later manifestations of normal metamorphosis in partially metamorphosed larvae. Two partially metamorphosed larvae were fixed and sectioned four days after the initiation of high K^+ seawater induced metamorphosis. Both of these larvae showed suggestions of cuticle deposition by the pedicle. In order to make this point with certainty, it would be necessary to do a study of these larvae at an electron microscope level of resolution. I did not observe any indication of mouth formation in these partial larvae; however, they may not have been cultured long enough.

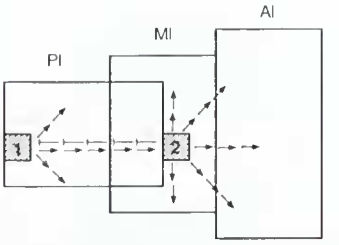

Figure 8. Diagrammatic view of a swimming larva with an apical lobe (AL), mantle lobe (ML), and pedicle lobe (PL). At the distal end of the pedicle lobe there is a postulated center composed of cells (1) which may use voltage-dependent calcium channels to transduce a substrate mediated metamorphic signal. This center sends a stimulatory metamorphic signal to other cells in the larva including center (2) which functions via voltage-dependent calcium channels that acts as a secondary metamorphic center. Here this center is shown in the mantle lobe but it could be any place outside of the distal end of the pedicle lobe. The cells of this secondary metamorphic center send a stimulatory metamorphic signal to other cells of the larva and an inhibitory signal to the cells that transduce the substrate mediated metamorphic signal turning off the metamorphic stimulus from these cells. This model accounts for the experiments described in this paper.

A variety of factors probably play a role in generating the partial metamorphosis phenotype. In larvae that have been reared for a number of days in a sterile environment intrinsic maturational changes may occur so that various parts of the metamorphosis signaling pathway or cells that respond to the signaling pathway may be activated. If the postulated distal pedicle lobe substrate receptor cells were activated, an aged larvae may undergo normal metamorphosis. This happened in a small percentage of cases (Table II). If cells that are part of the metamorphic pathway that reside outside of the distal region of the pedicle lobe are activated or if cells that will form the protegulum are activated, a larva that shows the partial metamorphosis phenotype would be generated. There is evidence that in some species with a bathy-pelagic life cycle that larvae which do not see an appropriate metamorphic cue in nature will metamorphose or partially metamorphose and still continue a pelagic existence (Thorson, 1946; Paine, 1963).

The mechanics of mantle lobe reversal during metamorphosis are not understood. There is a pair of muscles that insert in the mantle lobe and the pedicle lobe that are thought to contract during metamorphosis causing the mantle lobe to flip (Franzen, 1969; Long, 1964). Substrate adhesion by the pedicle lobe may be necessary for these muscles to contract or to cause the pedicle lobe to be compressed in an appropriate way as the muscles contract so that the mantle lobe is reversed. The production of larvae that show partial metamorphosis following substrate detachment could occur because protegulum formation is activated even though mantle reversal is inhibited. The small number of cases where partial metamor-

phosis occurs following the culture of larvae in the presence of substrates that induce metamorphosis can be explained in this way. Partially metamorphosed larvae and the conditions where they are formed provide an insight into the normal metamorphosis process.

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